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Mechanism of congenital lymphocytes and intestinal immunity regulated by gut microbial metabolites via metabolite-sensing receptor Ffar2

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ABSTRACT

Objective: The DSS was utilized to construct colitis model of mouse. The colitis mice were colonized with gut microbiota. The effects of gut microbial metabolites on colitis were studied. The mechanisms of gut microbial metabolites to improve intestinal immunity were also further explored. **Methods:** The male BALB/c mice were selected to construct colitis mouse model with DSS and colonized with gut microbiota. The content of short-chain fatty acids in intestinal metabolites of mice during modeling were detected with GC-MS. After the mice were sacrificed, the colon tissue was stained to observe the colitis in different groups of mice. The contents of IL-22 and IL-17 in colon tissue was determined with ELISA method. To study the mechanism of relieving colitis, qRT-PCR and western blotting were used to study the horizontal of Ffar2 gene and pSTAT3 and pAKT in colon tissue, respectively. The congenital lymphocytes were isolated and purified, and the migration ability of the congenital lymphocytes was examined by cell scratch plane migration test. **Results:** The colonization of the gut microbiota had significant effects on the contents of short-chain fatty acids in the intestinal metabolites of colitis mice, of which the effect on the content of acetic acid and butyric acid was more significant. The colonization of gut microbiota could effectively relieve colitis in mice and effectively promote the secretion of IL-22 in colon tissue. Studying the remission mechanism indicated that colonization of gut microbiota with colitis could effectively promote the expression of Ffar2 gene in colon tissue and increase the expression of pSTAT3 and pAKT protein. The migration ability of the lymphocyte was significantly upregulated in the model group compared with the other groups, demonstrating that DSS can effectively activate the lymphocyte; The migration of congenital lymphocyte in the experimental group was significantly alleviated than that in the model group, but it was up-regulated than that in the positive control group, and the colonic tissue of the positive control group was

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similar to that of the normal group. **Conclusion:** The short-chain fatty acids in the intestinal flora metabolites can promote the gene expression of the metabolite-sensitive receptor Ffar2. The effective combination of short-chain fatty acids and Ffar2 receptors can promote the phosphorylation of STAT3 and AKT proteins, effectively promote the secretion of IL-22 in intestinal ILC3 cells, alleviate colitis in mice, and thereby improve their intestinal immune function.

KEYWORDS

ILC3s; Intestinal immunity; Gut microbial metabolite; Ffar2

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1. Introduction

A large number of microorganisms inhabit the intestine and co-evolve with the host to form a mutually beneficial relationship [1]. These symbiotic microorganisms are called microflora and can be regarded as a multicellular organ, which communicates with and influences the host in many ways [2]. Intestinal microflora plays meaningful role in the formation of host system immune function [3]. New evidence shows that microflora affects host metabolism [4]. Microbial communities may affect host physiology by producing specific metabolites or inducing host responses to symbiont-related molecular patterns [5, 6]. The best microbial metabolite in this field is SCFAs. SCFAs is produced by fermentation of indigestible polysaccharides by intestinal bacteria. SCFAs exist in high concentration in the intestine, with acetate as the main component [7]. SCFAs is related to the maturation of host immune system, including inducing peripheral regulatory T cells, preventing infection, regulating metabolic rate and energy homeostasis [8-11]. SCFAs can activate metabolically sensitive receptor -G protein-coupled receptor (GPCRs) [12]. Ffar2, also known as GPR43, is an SCFA-sensitive G protein-coupled receptor, which plays an immunomodulatory role and function in intestinal homeostasis and inflammatory regulation [13].

Congenital lymphocytes (ILCs) play an important role in immunity, tissue homeostasis. ILCs can be divided into three types: type 1 congenital lymphocytes (ILC1s), type 2 congenital lymphocytes (ILC2s) and type 3 congenital lymphocytes (ILC3s), among which ILC3s can manufacture IL-22 and/or IL-17[14, 15]. ILC3s is enriched in intestinal tract, and maintains the balance in vivo by regulating the development of lymphoid tissue [16]. ILC3s processes signals from soluble media in the microenvironment of other cells and tissues [17]. Environmental signals regulate ILC3s through intracellular receptors. Bacterial metabolites and dietary components combine with Ahr to encourage the proliferation of ILC3 and the secretion of cytokines [18]. RA and RARs develop IL-22 through ILC3s [19].

At present, the effect of intestinal flora metabolites on the proliferation and occurrence of congenital lymphocytes and its mechanism with intestinal immunity are not clear. Therefore, in this study, mice were used as animal models, and a mouse colitis model was established by DSS. By colonization of intestinal flora, the contents of SCFAs metabolites, colon inflammation in mice, the contents of IL-22 and IL-17 in mouse colon tissue, the expression of metabolite sensitive receptor Ffar2 gene and AKT and STAT3 signaling pathway related proteins were studied, and the mechanism of intestinal flora metabolites regulating congenital lymphogenesis and intestinal immunity was explored.

2. Materials and methods

2.1. Experimental animals

Clean male BALB/c mice aged 6-8 months were selected and purchased from Peking University Animal Experimental Center. BALB/c mice were 6-8 weeks old and weighed (20±2) g. Living conditions required 22±1°C, indoor relative humidity 50%, normal drinking and eating every day. The mice were fed for a week to adapt to the environment.

2.2. Reagents and instruments

Agilent 6890N gas chromatography system (Agilent, California, USA) was equipped with FID detector and HP-INNOWAX column (19090-213, Agilent, USA); Olympus-Cx31 microscope (Olympus, Japan); Inverted microscope OLYMPUS, Japan); LACTOBACILLUS ACIDOPHILUS) KLDS; Sodium dextran sulfate (DSS) and short-chain fatty acid standards (acetic acid, propionic acid, n-butyric acid, isobutyric acid and valeric acid) were all obtained from Sigma.

Detection kit for IL-22 and IL-17 (Sigma, USA); Sodium acetate and sodium propionate (Sigma, USA); Trizol reagent, PrimescriptTM RT kit (Takara, Japan), real-time SYBR Green qPCR Master Mix; The q-RT PCR misture kit (Sigma, USA); β-actin (Santa Cruz, USA), horseradish peroxidase (HRP) and secondary antibody (Santa Cruz, USA); PAKT and pSTAT3 antibodies (Santa Cruz, USA) were used.

2.3. Animal experiments

Sterile mice were divided into groups and fed continuously, with 10 mice in each group. Normal group: normal feeding, with physiological saline as the negative control. Model group: given water containing DSS for one week, normal drinking for two weeks, normal diet, repeating three cycles; experimental group: given water containing DSS for one week, normal drinking for two weeks, normal diet, taking it as a DSS cycle, repeating three cycles; Positive control group: water containing DSS(3% w/v), sodium acetate (150 mmol/L) and sodium propionate (150 mmol/L) was given at the same time for one week, drinking water normally for two weeks, eating normally and repeating three cycles. During the experiment, we should carefully observe the weight and defecation of mice and make records. After the third DSS cycle, the mice were killed.

2.4. Analysis of SCFAs content in intestinal metabolites of mice

Collect the feces of mice before DSS administration (DSS1, DSS2, DSS3) and before death in each cycle, prepare a suspension with a certain concentration with distilled water, mix evenly, ultrasonic treat for 25 min, let stand for 20 min, mix evenly again, then centrifuge, take the supernatant. The supernatant was filtered by 0.22 μm microporous membrane, and then the content of short-chain fatty acids in the supernatant was determined by Agilent 6890N gas chromatography system (Agilent, California, USA) equipped with FID detector and HP-INNOWAX column (19090-213, Agilent, California, USA): the column temperature was 240℃, The detector temperature was 240℃, the throughput was 0.1 μL, the split ratio was 1:10, the carrier gas He, the flow rate was 19.0 mL/min, and the inlet temperature was 240℃.

2.5. Colon inflammation in mice

After the mice were killed, the colon was washed with PBS, and the cells were fixed, embedded in paraffin, sectioned and stained with HE. Then the inflammation of the colon in mice was observed by inverted microscope.

2.6. Determination of IL-22 and IL-17 in colon tissue cells of mice

A certain amount of mouse intestinal tissue was homogenized, centrifuged, and the contents of related cytokines IL-22 and IL-17 in the supernatant were determined by ELISA.

2.7. Determination of Ffar2 expression in colon tissue cells of mice

RT-PCR was used to test the horizontal of Ffar2 mRNA in mouse intestinal tissue cells. Take a certain amount of mouse colon tissue, homogenize it with Trizol reagent, and determine the total RNA under aseptic conditions according to the instructions of RNA extraction kit, and determine its concentration by spectrophotometry, so that the extracted RNA OD260/OD280 was between 1.6 and 1.8. The extracted RNA was subpackaged and stored in the refrigerator at -80℃. The corresponding gene primer sequences are shown in the table below: Ffar2 upstream primer: AATCACAGGAAACGGGAAGCC, and downstream primer: GTCTGGGGTCATTCTCCTTGG; The upstream primer of β-actin was CGCAAAGACCTGTATGCCAAT, and the downstream primer was GGGCTGTGATCTCCTTCTGC. CDNA was used as template, β-actin was used as internal reference,.and referring to the instructions of Kangwei

Century Quantitative Real-time PCR Misture kit, PCR amplification was tested by using a real-time fluorescent PCR instrument, and the mRNA expression of Ffar2 was analyzed.

2.8. Analysis of AKT and STAT3 signaling pathway related proteins expression in mouse colon tissue cells

Take wound tissue, add cell lysate to homogenize for 25 min, centrifuge at 10000 r/min for 20 min, take supernatant, and measure protein concentration with BCA kit. The same amount (40 μg) of protein was loaded on the 12% sodium dodecyl sulfate-polyacrylamide gel, and it was electrophoresed at a constant voltage of 70 V, then at 120 V, and finally transferred to the polyvinylidene fluoride membrane at 300 mA. The membrane was sealed with 5% skim milk at 20°C, and then the primary antibody was added to incubate overnight. It was washed in TBS for 3 times, covered with appropriate HRP-bound secondary antibody (1:5000), and observed by ECL on X-ray film. The optical density ratio of bands was recorded to evaluate the expression level of each protein.

2.9. Analysis of congenital lymphocyte migration in mice

After the mice were killed, the colonic tissues were taken to isolate and purify the lymphocyte, and the migration ability of the lymphocyte was examined by cell scratch plane migration test. The cells were inoculated on the slide. After the routine culture, the sterile Eppendorf Tip made a 3 mm linear scratch on the slide. The cells were washed repeatedly with PBS buffer and incubated in the incubator for 24 hours. Five randomly selected high power fields were used to count the number of cells migrating to the scratch area, and the number of migrating cells was used to indicate the migration activity of cells. The experiment was repeated six times.

2.10. Statistical analysis

Data in this study were analyzed by SPSS20.0 statistical software (IBM, USA) , mean ± standard deviation (±s) was used for measurement data, and independent sample t-test was used for comparison between groups Percentage (%) was used to represent the count data, and $\chi 2$ analysis was used to analyze the comparison between groups; $P<0.05$ represented the statistical significance.

3. Results

3.1. Analysis of SCFAs content in intestinal metabolites of mice

The feces of mice before the first administration of DSS (DDS1), the second administration of DSS (DDS2), the third administration of DSS (DDS3) and before death were collected. The analysis of total short-chain fatty acid content was shown in Figure 1, and the total short-chain fatty acid content in the normal group remained stable. The content of total short-chain fatty acids in the model group decreased continuously with the increase of feeding time. The content of total short-chain fatty acids in the experimental group and the positive control group decreased significantly after feeding DSS water for the first time, and increased significantly with the increase of feeding time in the later period. By comparing the contents of total short-chain fatty acids in intestinal metabolites of mice before execution, it can be found that the model group was significantly lower than other groups, the experimental group was significantly higher than the normal group, and the positive control group was significantly higher than the experimental group. The analysis of acetic acid content in intestinal metabolites of mice was shown in Figure 2. The acetic acid content of normal group had no significant change with the raise of feeding time. The acetic acid content in the model group decreased significantly with the increase of feeding time. The acetic acid content in the

experimental group decreased significantly after feeding DSS water for the first time, and then raised significantly. The content of acetic acid in the positive control group increased with the increase of feeding time. Comparing the contents of acetic acid in intestinal metabolites of mice before execution, it can be found that the model group was significantly lower than other groups, and there was no significant difference between the experimental group and the normal group, but it was significantly lower than the positive control group. The analysis of propionic acid content in intestinal metabolites of mice was shown in Figure 3. The propionic acid content of normal group had no significant change with the raise of feeding time. Propionic acid content in model group reduecd significantly with the increase of feeding time; The propionic acid content in the experimental group and the positive control group decreased significantly after feeding DSS water for the first time, and then increased significantly. Comparing the content of propionic acid in intestinal metabolites of mice before execution, it can be found that the model group was significantly lower than other groups, and there was no significant difference between the experimental group and the normal group, but it was significantly lower than the positive control group. The contents of butyric acid, isobutyric acid and valeric acid in intestinal metabolites of mice were shown in Figure 4, Figure 5 and Figure 6, respectively. The contents of butyric acid, isobutyric acid and valeric acid in normal group had no significant changes with the increase of feeding time. The contents of butyric acid, isobutyric acid and valeric acid in model group decreased significantly with the increase of feeding time. The contents of butyric acid, isobutyric acid and valeric acid in the experimental group and the positive control group decreased after the first feeding of DSS water, and then increased to no difference with DSS1. Comparing the contents of butyric acid, isobutyric acid and valeric acid in intestinal metabolites of mice before execution, it can be found that the model group was significantly lower than other groups, and there was no significant difference between normal group, experimental group and positive control group. To sum up, the colonization of intestinal flora had a significant effect on the content of short-chain fatty acids in intestinal metabolites of colitis mice, especially on the content of acetic acid and butyric acid.

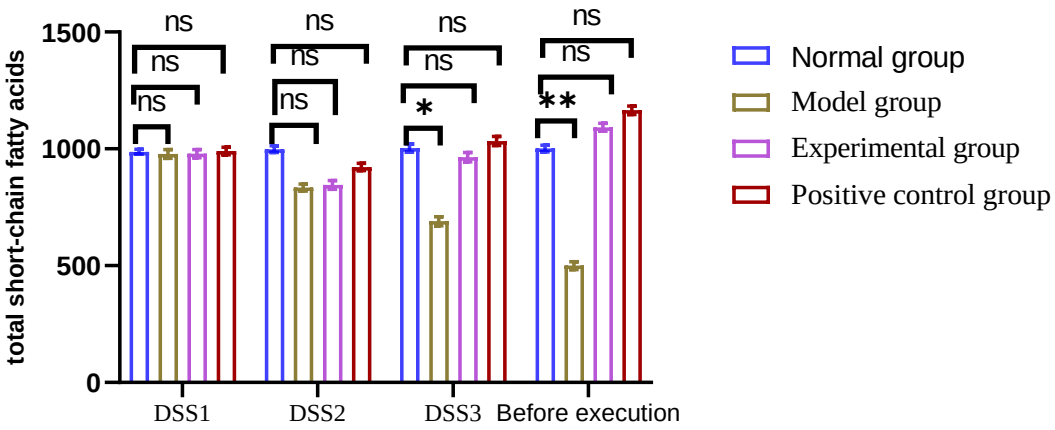

Figure 1. Analysis of total short-chain fatty acids in intestinal metabolites of mice (±s, n=10, mmol/L)

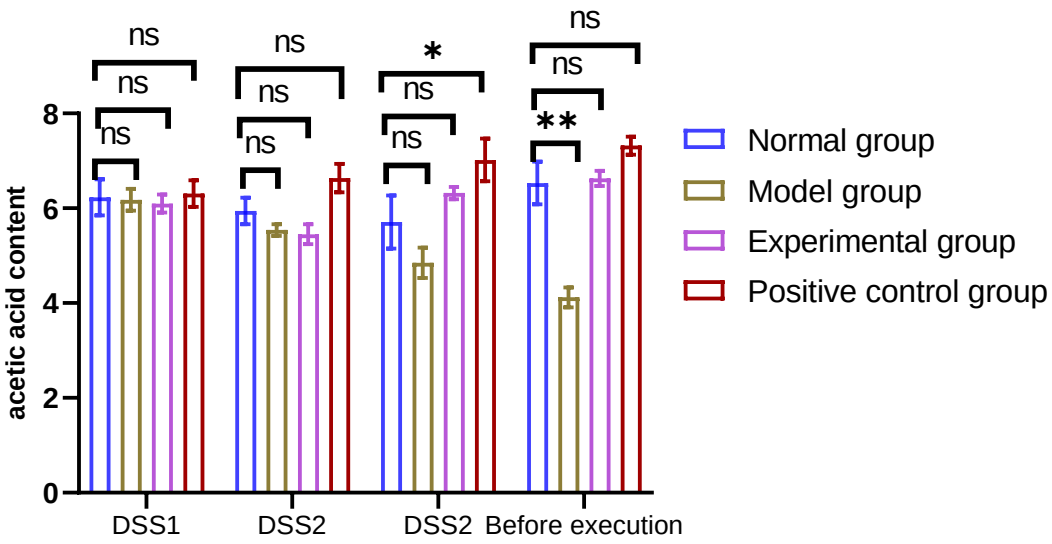

Figure 2. Analysis of acetic acid content in intestinal metabolites of mice (±s, n=10, mmol/L)

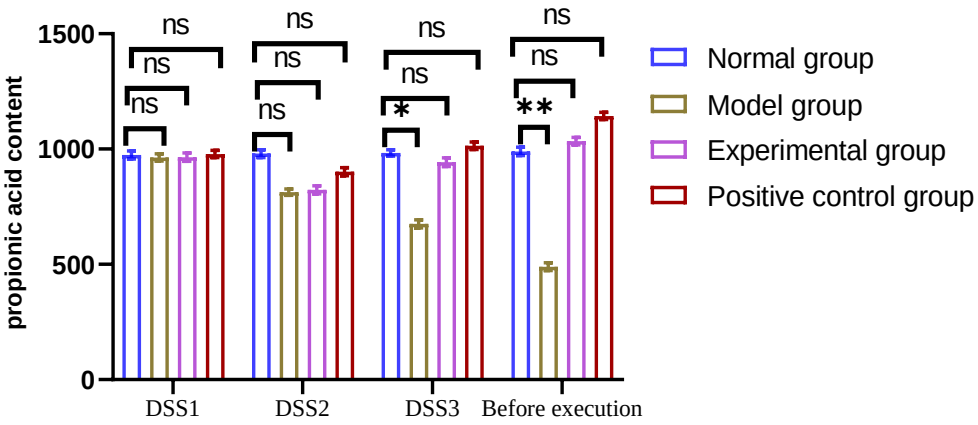

Figure 3. Analysis of propionic acid content in intestinal metabolites of mice (±s, n=10, mmol/L)

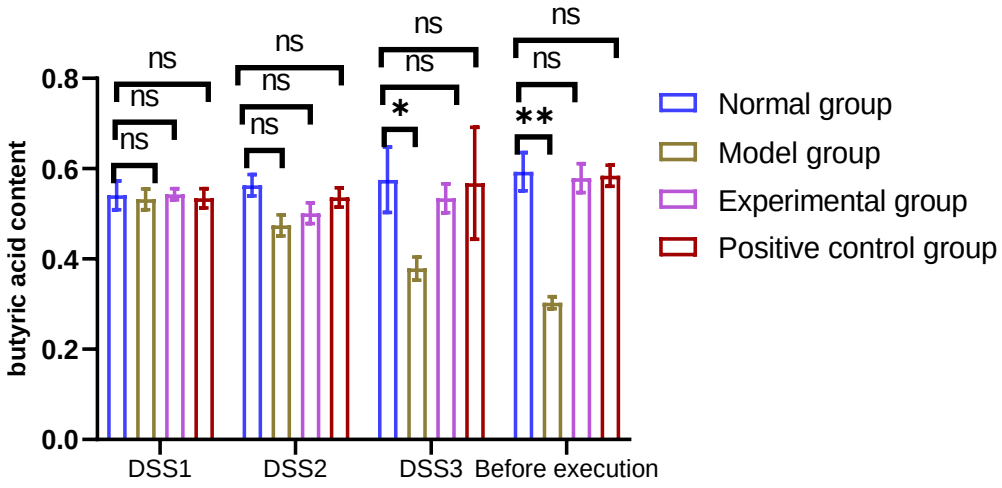

Figure 4. Analysis of butyric acid content in intestinal metabolites of mice (±s, n=10, mmol/L)

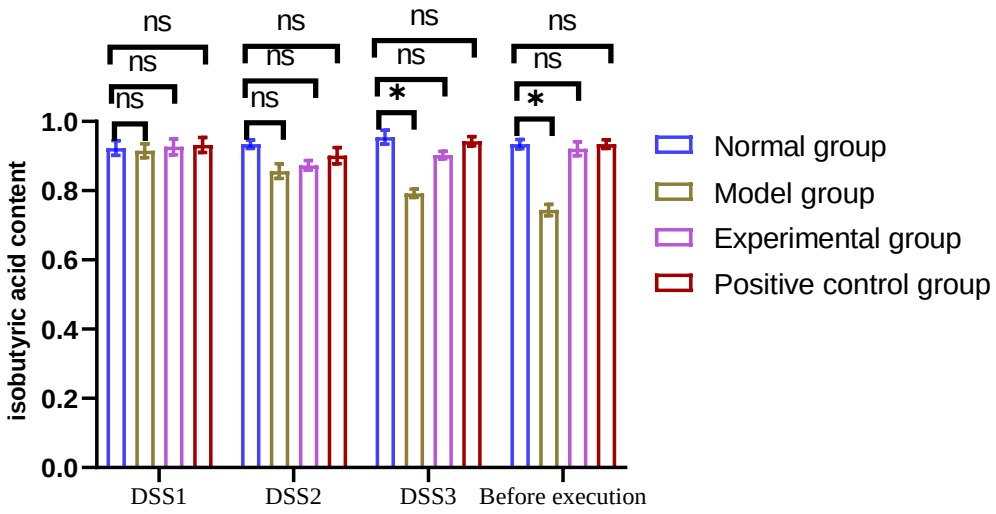

Figure 5. Analysis of isobutyric acid content in intestinal metabolites of mice (±s, n=10, mmol/L)

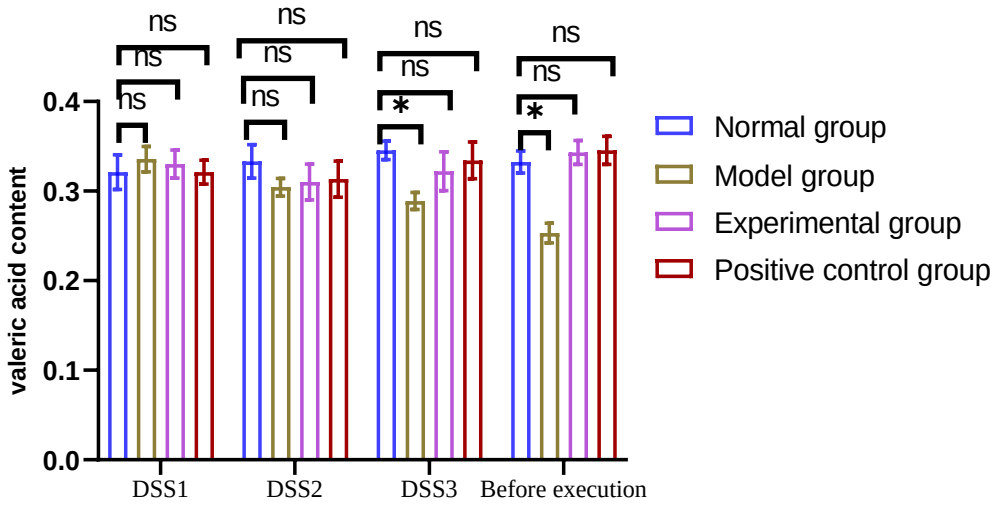

Figure 6. Analysis of valeric acid content in intestinal metabolites of mice (±s, n=10, mmol/L)

3.2. Analysis of Colon Inflammation in Mice

After the mice were killed, their colon tissues were taken and stained. As shown in Figure 7, the colitis in the model group was significantly more serious than that in other groups, which proved that DSS could effectively induce colitis in mice. Colitis in the experimental group was significantly relieved than that in the model group, but it was more serious than that in the positive control group, and the colon tissue in the positive control group was similar to that in the normal control group. It can be concluded from the above results that colonization of intestinal flora can effectively alleviate colitis in mice and improve their intestinal immunity.

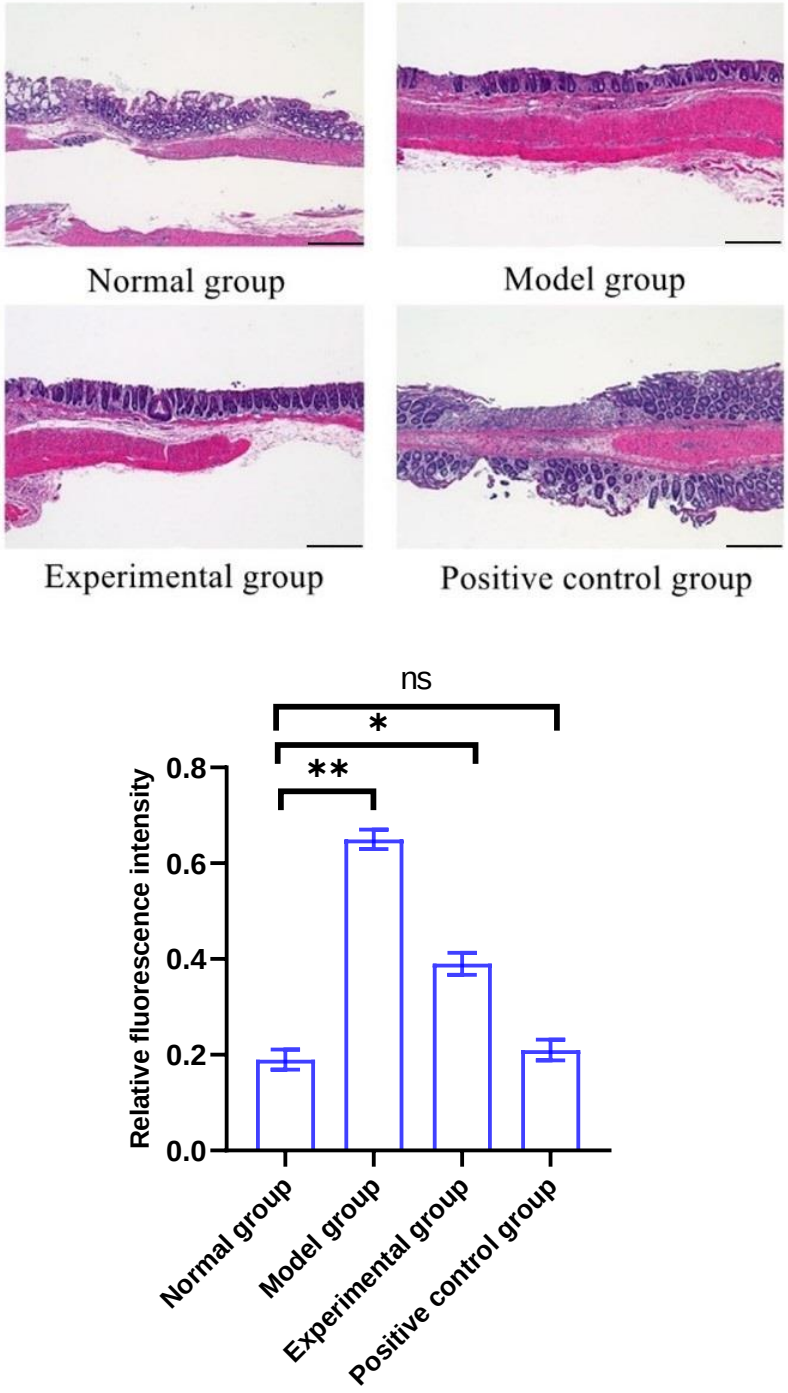

Figure 7. Analysis of intestinal inflammation in mice.

3.3. Analysis of the contents of IL-22 and IL-17 in colon tissue cells of mice

IL-22 and/or IL-17 are the key cytokines of IL-C3 function and play an momentous role in intestinal immunity [20]. Therefore, the contents of IL-22 and IL-17 in intestinal tissue cells were analyzed. As shown in Figure 8, the expression of IL-22 in normal group was significantly higher than model group, while that in experimental group was significantly higher than normal group, but significantly lower than that in positive control group. There was no significant distinguish in the contents of IL-17 in each group. It can be concluded that colonization of intestinal

flora in mice with colitis can effectively promote the secretion of IL-22 in intestinal IL-3 cells, relieve colitis in mice and improve their intestinal immune function.

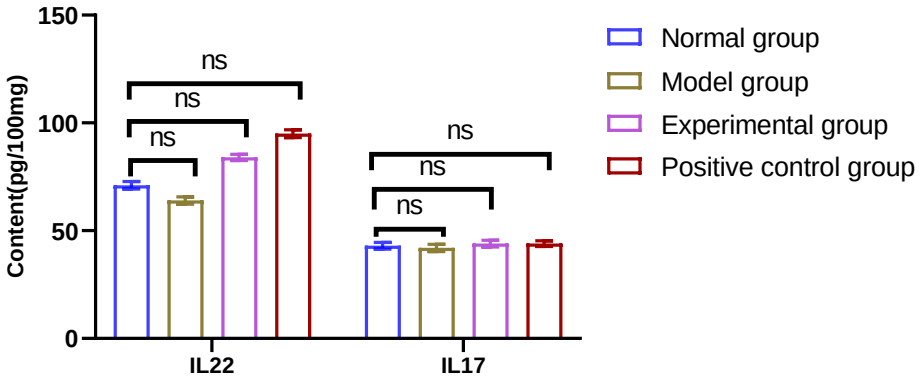

Figure 8. Content analysis of IL-22 and IL-17 in intestinal cells of mice.

3.4. Analysis of gene expression of Ffar2 in intestinal tissue cells of mice

QRT-PCR was used to analyze the gene expression of Ffar2 in mouse colon tissue, and the results were shown in Figure 9. The gene expression of Ffar2 in the model group was significantly lower than the control group, indicating that colitis can reduce the gene expression of Ffar2. The gene expression of Ffar2 in the experimental group was significantly higher than the normal group and model group, but significantly lower than that in the positive control group, which indicated that colonization of intestinal flora in colitis mice could effectively encourage the gene expression of Ffar2 in colon tissue.

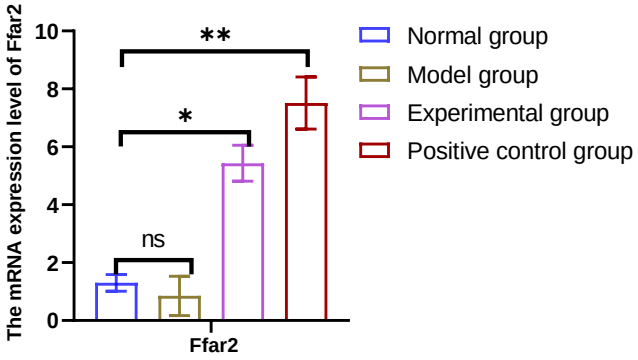

Figure 9. Gene expression analysis of Ffar2 in mouse colon tissue.

3.5. Analysis of the expression of STAT3 signaling pathway-related proteins in intestinal tissue cells of mice

The expression levels of pSTAT3 and pAKT proteins in mouse colon tissue cells were determined by Western blotting, and the results were shown in Figure 10. The expression levels of pSTAT3 and pAKT proteins in colon tissue of mice in model group were significantly lower than control group. The expression of pSTAT3 and pAKT protein in colon tissue of experimental mice was significantly higher than normal group and model group, but significantly lower than positive control group, which indicated that the colonization of intestinal flora in colitis mice could effectively promote the expression of pSTAT3 and pAKT protein in colon tissue.

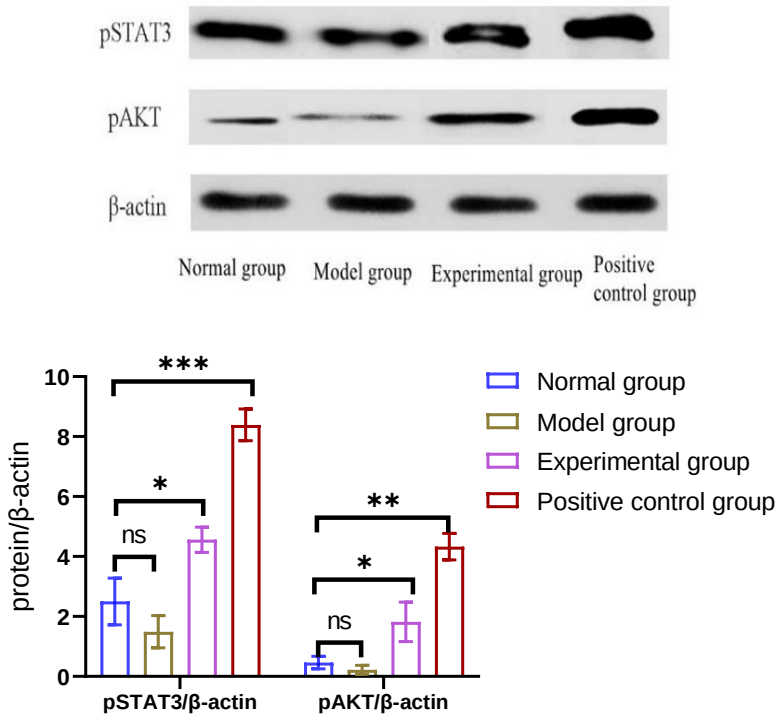

Figure 10. Analysis of the expression of pSTAT3 and pAKT proteins in mouse colon tissue cells.

3.6. Analysis of congenital lymphocyte migration in mice

After the mice were killed, the colonic tissues were taken to isolate and purify the lymphocyte, and the migration ability of the lymphocyte was examined by cell scratch plane migration test. As shown in Figure 11, the migration ability of the lymphocyte was significantly upregulated in the model group compared with the other groups, demonstrating that DSS can effectively activate the lymphocyte; The migration of congenital lymphocyte in the experimental group was significantly alleviated than that in the model group, but it was up-regulated than that in the positive control group, and the colonic tissue of the positive control group was similar to that of the normal group. From the above results, it can be concluded that colonization of intestinal flora can effectively activate the activity of congenital lymphocyte.

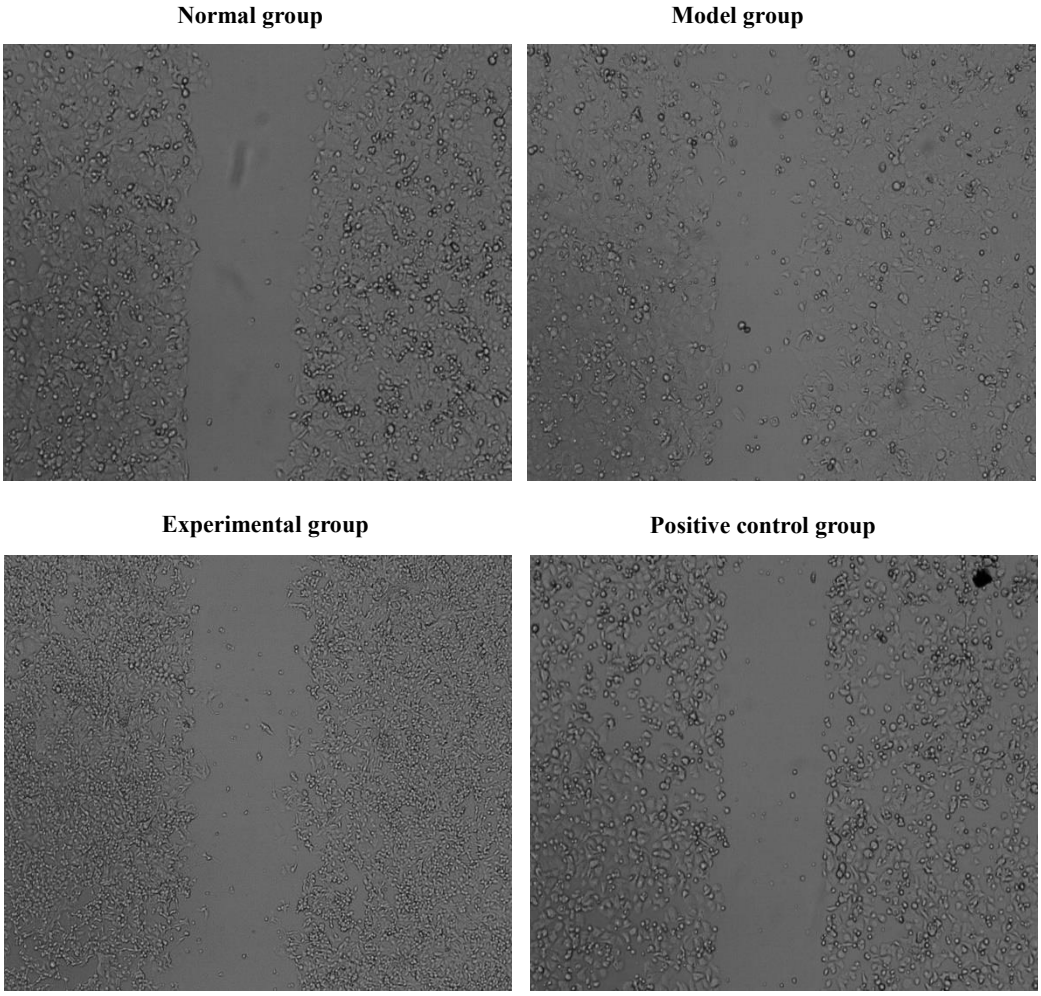

Figure 11. Analysis of congenital lymphocyte migration in mice.

4. Discussion

The mice were modeled by DSS to obtain colitis mice, and the intestinal flora was colonized to explore the mechanism of intestinal flora metabolites regulating congenital lymphopoiesis and intestinal immunity. It was concluded that colonization of intestinal flora had a significant effect on the content of short-chain fatty acids in intestinal metabolites of colitis mice, especially on the content of acetic acid and butyric acid. Colonization of intestinal flora can effectively alleviate colitis in mice, effectively promote the secretion of IL-22 in colon tissue and improve intestinal immunity. By studying its remission mechanism, it was concluded that giving colonized intestinal flora to colitis mice can effectively promote the gene expression of Ffar2 in colon tissue and increase the expression of pSTAT3 and pAKT proteins.

Intestinal microflora plays momentous role in forming the immune function of the host system and can affect the metabolic function of the host. SCFAs is an important metabolite of intestinal flora, which is closely related to the maturation of host immune system. ILC3s are enriched in the intestine, and maintain the homeostasis by regulating the development of lymphoid tissue, the inhibition of symbiotic bacteria, tissue repair, host defense and adaptive immunity, in which ILC3s can generate IL-22 and/or IL-17 [16]. According to the literature, SCFAs can activate Ffar2, and acetic acid and propionic acid are the strongest activators [21]. Ffar2 plays an obvious role in intestinal immunity. It promotes the phosphorylation of STAT3 and AKT proteins through STAT3 and AKT, and then

regulates the proliferation of IL-3 cells in colon tissue and the secretion of IL-22 [22]. It can be concluded that short-chain fatty acids in intestinal flora metabolites can promote the gene expression of metabolite sensitive receptor Ffar2, and the effective combination of short-chain fatty acids and Ffar2 receptor can encourage the phosphorylation of STAT3 and AKT proteins, thus effectively promoting the secretion of IL-22 in intestinal ILC3 cells, alleviating colitis in mice, and further improving their intestinal immune function. In this paper, the mechanism of intestinal flora metabolites regulating congenital lymphogenesis and intestinal immunity through metabolite sensitive receptor Ffar2 was clarified, which provided a scientific explanation for intestinal flora to provide intestinal immunity from a new perspective.

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This research received no external funding.

Conflict of interest

The author claims that the manuscript is completely original. The author also declares no conflict of interest.

Author contributions

Conceptualization, data curation, formal analysis, funding acquisition, investigation, methodology, project administration, resources, software, supervision, validation, visualization, writing–original draft, and writing–review & editing are conducted by solely by the first author.

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